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The Effect of Ionizing Radiation on mRNA Levels of the DNA Damage Response Genes *Rad9, Rad1* and *Hus1* in Various Mouse Tissues

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***Rad9, Rad1* and *Hus1* are essential genes conserved from yeast to humans. They form a heterotrimer complex (9-1-1 complex) that participates in the cell cycle checkpoint activation and DNA damage repair in eukaryotic cells. *Rad9, Rad1* and *Hus1* deficient cells are hypersensitive to ionizing radiation and mouse cells deleted for anyone of the three genes are highly sensitive to the killing by gamma rays. We propose that ionizing radiation-induced transcription of these genes is a mechanism by which cells respond to radiation-induced damage. In this study we used quantitative real-time RT-PCR(qPCR) to analyze the mRNA levels of *Rad9, Rad1* and *Hus1* in various tissues isolated from mice that were either mock irradiated or exposed to 10 Gy gamma radiation. Our results indicated that the mRNA levels of *Rad9, Rad1* and *Hus1* genes were very different among these tissues, and we found high natural levels of mRNA in the spleen, lung, ovary and testis of mice before exposure to radiation. The mRNA levels of the three genes were well correlated across these tissues, being high, medium or low in each of the tissues simultaneously. The mRNA levels of the three genes were analyzed at 2, 6, 12, 24 and 48 h after irradiation. In most tissues *Rad9* was strongly induced at 2 and 12 h time points and *Hus1* was strongly induced at 2, 12 and 48 h time points, but *Rad1* was minimally induced in most of the tissues with the exception of slightly higher levels in the heart and lung tissues at the 48 h time point. These results suggest that the regulation mechanisms for the mRNA levels of the three genes in response to ionizing radiation are complex and not well orchestrated. We also detected the induction of Rad9 and Hus1 proteins in the heart and liver of the animals after irradiation, and found that Rad9 protein levels were highly induced in both the heart and liver, while the Hus1 protein levels were significantly induced only in the liver, suggesting that Rad9 and Hus1 protein levels are not regulated in a coordinated manner in response to irradiation. We then went on to measure the mRNA levels of the three genes and the Rad9 and Hus1 protein levels in the mouse liver cell line (NCTC 1469) in response to irradiation *in vitro*. All three genes in the cultured cells were minimally induced at mRNA level, obviously different from the highly dynamic induction in liver. Rad9 and Hus1 were significantly induced at the protein level, but the induced Rad9 protein levels were higher than the Hus1 levels. Taken together, the good correlation of the mRNA levels of *Rad9, Hus1* and *Rad1* genes across different tissues isolated from the animals that were mock irradiated and the lack of correlation in mRNA as well as protein levels after irradiation suggest that the 9-1-1 complex has evolved to play various physiological roles in tissues rather than dealing with high doses of gamma radiation or other genotoxic agents.**

INTRODUCTION

Ionizing radiation (IR) is ubiquitous in the environment and widely used in radiotherapy and for diagnostic purposes. IR is also potentially hazardous to humans because it is also a mutagen and a carcinogen (*1*). To determine its potential health risks for humans or animals, an understanding of how tissue responded to IR is of vital importance. Radiation exposure induces damage that can be detected by cellular systems, triggering cell cycle arrest (*2*) and DNA repair pathways (*3*). In response to radiation exposure, a variety of DNA repair proteins detect and repair various types of DNA lesions such as double-strand breaks and replication errors. These DNA repair pathways include the base excision repair (BER), nucleotide excision repair (NER) and mismatch repair (MMR) pathways (*4*). If the lesions cannot be repaired, the cell can enter into permanent

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arrest or can die by programmed cell death pathways such as apoptosis (*4–6*).

In recent years, a number of radiation-induced biomarkers have been developed (*7*). These radiation biomarkers include gene expression changes (*8, 9*), DNA mutations (*10*) and protein expression changes (*11*). *Rad9, Rad1* and *Hus1* genes are conserved from yeast to humans, that form a heterotrimer complex called the 9-1-1 complex (*12–15*). Each component of the 9-1-1 complex plays an important role in the maintenance of genome integrity and in resistance to genotoxic stresses (*16–29*), although there has been no direct experimental evidence that the 9-1-1 complex itself plays a key role in resistance to large scale DNA lesions induced by exposure to high doses of ionizing radiation or other genotoxic stresses. Because of the availability of high quality antibodies for detecting Rad9 protein, many more articles on Rad9 have been published than on Hus1 and Rad1. In clinical tumor samples, aberrant *Rad9* expression has been associated with prostate (*30*), breast (*31*), lung (*32*), thyroid (*33*), skin (*20*) and gastric cancers (*34*). There are high levels of *Rad9* expressions in lung, thyroid, breast and prostate cancers and diminished levels of *Rad9* expression in skin papilloma and gastric carcinoma. Although radiation-induced gene expression changes in cells (*35*) have been widely studied, data on the induction of *Rad9, Rad1* and *Hus1* in tissues postirradiation is lacking. It has been reported that Rad9, Rad1 and Hus1 became more firmly anchored to cell nuclear components after gamma irradiation, consistent with their function in DNA damage-activated checkpoint signaling pathways (*36*). However, the effects of gamma irradiation on the *Rad9, Rad1* and *Hus1* expression *in vivo* after whole body irradiation on *Rad9, Rad1* and *Hus1* gene expression in mice has not been reported and may be different from those in cultured cells.

The underlying mechanisms for the regulation of *Rad9, Rad1* and *Hus1* genes remain unclear. While it is widely believed that Rad9, Rad1 and Hus1 usually form the 9-1-1 complex to participate in DNA repair, several recent studies have shown that the contributions of *Rad9, Rad1* and *Hus1* genes to DNA repair can sometimes be independent of their roles in the 9-1-1 complex (*22, 37*). Hence, this study focused on investigating any potential correlations of *Rad9, Rad1* and *Hus1* gene expression in various normal mouse tissues and the regulation of theses genes in the tissues after gamma-radiation exposure. To the best of our knowledge, investigation of the expression responses of *Rad9, Rad1* and *Hus1* genes to gamma irradiation in either isolated cells or in animal tissues is novel. In this study, we attempted to quantify changes in the expression of *Rad9, Rad1* and *Hus1* genes of whole body gamma-irradiated mice using qPCR analysis, and studied various tissues isolated from the irradiated animals. For this study we used qPCR because it is considered to be an accurate quantitative method for gene expression analysis (*38*). Using qPCR analysis, we first analyzed the mRNA levels of *Rad9, Rad1* and *Hus1* in different normal mouse tissues, and then investigated the radiation-induced changes at the mRNA levels of *Rad9, Rad1* and *Hus1* genes in different tissues from the irradiated mice. Our results indicated that the mRNA levels of *Rad9, Rad1* and *Hus1* genes strongly correlated with various nonirradiated normal mouse tissues in both male and female mice, but these differences were not maintained within each of the these tissues after ionizing radiation. The results of this study suggest that the regulation mechanisms for the mRNA levels of *Rad9, Rad1* and *Hus1* genes in response to ionizing radiation exposure are different.

MATERIALS AND METHODS

Animals, Cells and Irradiation

A total of 11 male and 35 female, 6–8 weeks old, wild-type 129 mice were purchased from Vital River Laboratories Inc. (Beijing, China). In the first experiment, the tissues of 5 male and 5 female mice were dissected and collected to analyze the mRNA levels of the *Rad9, Rad1* and *Hus1* genes. Second, after a two day acclimatization period, the remaining 30 female mice were delivered to the Peking University Radiation Facility (Beijing, P.R. China), where they were exposed to 0 or 10 Gy of whole body gamma irradiation. The mice in group 1 served as the control group (0 Gy), and the mice in groups 2–6 were exposed to a single dose of 10 Gy radiation from a Co-60 source at a dose rate of 1 Gy/60 s. After exposure, the controls and irradiated mice were sacrificed by cervical dislocation at 2, 6, 12, 24 and 48 h postirradiation, respectively, and the tissues were dissected and immediately frozen in liquid nitrogen. The male mice were also used in a similar radiation exposure experiment. All animal procedures were approved by the Institutional Animal Care and Use Committee at the Institute of Biophysics, Chinese Academy of Sciences.

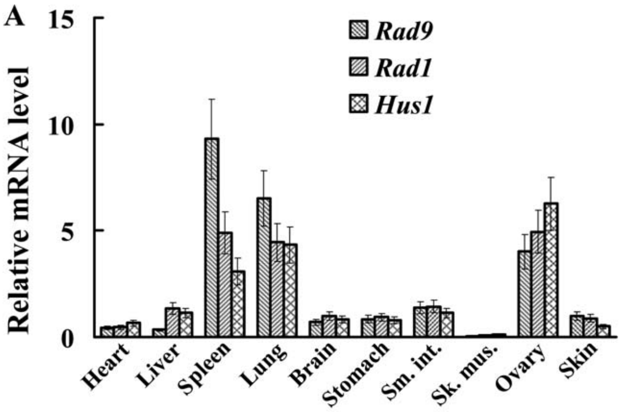
Mouse liver cells (NCTC 1469) were cultured and placed in a 5% CO_2 37°C cell incubator. The cells were irradiated with 0 or 10 Gy using the same procedure as described above for mouse irradiation.

RNA Isolation and Quantitative Real-Time RT-PCR Analysis

Total RNA was isolated from tissues of irradiated and sham-irradiated mice with the RNeasy Mini kit (QIAGEN, Hilden, Germany), as described by the manufacturer. A total of 2 μg RNA were reverse-transcribed in a 20 μL reaction volume to form cDNA using the SuperScript® First-Strand Synthesis System for Quantitative real-time RT-PCR (Invitrogen™, Carlsbad, CA). RT-PCR was performed using the StepOnePlus™ system (Applied Biosystems, Carlsbad, CA) with SYBR green reaction master mix (TaKaRa, Shiga, Japan) to label amplified DNA. A standard curve method of quantification was used to calculate the expression of target genes relative to the housekeeping gene GAPDH. Experiments were performed three times. The following primer pairs, which were synthesized by Biosune, Inc. (Beijing, China), were used for the PCR reactions:

Mouse *Rad9*: 5′-GCCTCATGCCCTCACTT-3′ and 5′-TGCTGTCTGCCTCCTCTT-3′;
Mouse *Rad1*: 5′-GCCCTATTTCAGGTTGT-3′ and 5′-TGCCCATCTTCATTTCT-3′;
Mouse *Hus1*: 5′-TCCCTGTCTTACCGTGTC-3′ and 5′-CTCCCTTTAGGTTTGCTT-3′;
Mouse GAPDH: 5′-ACCCAGAAGACTGTGGATGG-3′ and 5′-GGATGCAGGGATGATGTTCT-3′.

The PCR procedure was performed as follows: template denaturation at 95°C for 15 s, then 40 cycles of 95°C for 10 s, 60°C for 20 s and 72°C for 20 s. The RNA isolation from the mouse liver cells



B **Correlations**

		Rad9	*Rad1*	*Hus1*
Rad9	Pearson correlation	1	0.904**	0.696*
	Sig. (2-tailed)		0.000	0.025
	N	10	10	10
Rad1	Pearson correlation	0.904**	1	0.927**
	Sig. (2-tailed)	.000		.000
	N	10	10	10
Hus1	Pearson correlation	0.696*	0.927**	1
	Sig. (2-tailed)	0.025	0.000	
	N	10	10	10

FIG. 1. The expressions of *Rad9, Rad1* and *Hus1* genes in female 129 mouse tissues (heart, liver, spleen, lung, brain, stomach, small intestine, skeletal muscle, ovary and skin). The expressions were measured by quantitative real-time PCR. Panel A: The mRNA levels of *Rad9, Rad1* and *Hus1* genes in various normal mouse tissues. The expression levels in spleen, lung and ovary were significantly higher than the other seven tissues. Data are shown as ratios relative to the expression of GAPDH and are normalized by average levels of the 10 tissues. Error bars represent standard deviations of three independent experiments. Sm.int. = small intestine; Sk.Mus. = skeletal muscle. Panel B: Correlation coefficient of expression levels of *Rad9, Rad1* and *Hus1* genes. The mRNA levels of *Rad9, Rad1* and *Hus1* genes showed statistically significant correlations in various normal mouse tissues, especially between *Rad1* and *Hus1*.

(NCTC 1469) and the RT-PCR for tissues were the same as described above.

Western Blotting

To prepare protein from mouse liver, approximately 30 µg of the liver was homogenized in lysis buffer. The lysate was then prepared in 1× SDS-sample buffer. Fifty µg of protein was resolved on 10% SDS-polyacrylamide gel electrophoresis (PAGE) gel, and then transferred to a polyvinylidene difluoride membrane (PVDF). The membrane was probed consecutively with primary and peroxidase-conjugated secondary antibodies, and the signal was detected using the Super Signal West Pico Chemiluminescence Substrate system (Pierce, Rockford, IL). The primary and secondary antibodies used in this study were: mouse anti-GAPDH (KangChen, China), mouse anti-RAD9 (BD Pharmingen™, San Diego, CA) and peroxidase-conjugated anti-mouse IgG (A9044, Sigma-Aldrich®, St. Louis, MO). The protein lysate preparation from the mouse liver cells (NCTC 1469) and their analysis were the same as above described for tissues.

Statistical Analysis

All statistical analyses were performed using statistical software package SPSS Version 10.0. The Student's *t* test was performed to determine statistical significance of the differences between the expressions of *Rad9, Rad1* and *Hus1*. In all the above analyses, a *P* value of <0.05 was considered statistically significant.

RESULTS

The mRNA Levels of Rad9, Rad1 and Hus1 Genes Were Well Correlated in Various Normal Mouse Tissues

Using qPCR analysis, we examined the mRNA levels of *Rad9, Rad1* and *Hus1* in 10 normal wild-type 129 mouse tissues (heart, liver, spleen, lung, brain, stomach, small intestine, skeletal muscle, ovary/testis and skin) of both sexes [Fig. 1 and Supplementary Fig. S1 (http://dx.doi.org/10.1667/RR13781.1.S1)]. Statistical analysis of our results demonstrated that there was good correlation between the mRNA levels of *Rad9, Rad1 and Hus1* genes in the various mouse tissues, especially between *Rad1* and *Hus1* [Fig. 1B and Supplementary Fig. S1B (http://dx.doi.org/10.1667/RR13781.1.S1)], and there was no difference in the mRNA levels of these genes between the male and female mice (Fig. 2), suggesting that the three genes are regulated in normal mouse tissues in a coordinated manner. Interestingly, the expression levels in the spleen, lung and ovary/testis were significantly higher than the other tissues [Fig. 1A and Supplementary Fig. S1A (http://dx.doi.org/10.1667/RR13781.1.S1)]. Understandably, the induced DNA breaks and their repair required for antibody formation in the blood cells of the spleen and that occurs in the gamete cells during meiosis of ovary/testis may explain the higher expression of repair genes such as *Rad9, Rad1* and *Hus1* in those two tissues (*24, 39–41*), but the higher expressions of these three genes in the lung is not well understood. The deviations of *Rad9* expression levels from *Rad1* and *Hus1* expression levels in the spleen and lung are major contributors to a weaker correlation between *Rad9* and *Rad1*/*Hus1* expression levels [Fig. 1 and Supplementary Fig. S1].

Responses of Rad9, Rad1 and Hus1 Genes to Gamma Irradiation Were Not Correlated in Each of the Same Mouse Tissues

The main purpose of this study was to better characterize the cellular response of tissues to gamma irradiation. Female mice were exposed to a whole body lethal dose (10 Gy) of gamma irradiation (*42*). The mRNA levels from these tissues were examined by qPCR at 2, 6, 12, 24 and 48 h after irradiation compared to sham-irradiated controls. The results showed prominent increase in the mRNA levels of *Rad9* and *Hus1* genes after irradiation at some time points (Fig. 3A and C), while the mRNA level of the *Rad1* gene was barely altered under the same conditions (Fig. 3B), indicating that the induction mechanisms of the three genes after a high dose of gamma radiation are different in most if

A Correlations

		Rad9 Female	*Rad9* Male
Rad9 Female	Pearson correlation	1	0.951**
	Sig. (2-tailed)		0.000
	N	9	9
Rad9 Male	Pearson correlation	0.951**	1
	Sig. (2-tailed)	0.000	
	N	9	9

B Correlations

		Rad1 Female	*Rad1* Male
Rad1 Female	Pearson correlation	1	0.982**
	Sig. (2-tailed)		0.000
	N	9	9
Rad1 Male	Pearson correlation	0.982**	1
	Sig. (2-tailed)	0.000	
	N	9	9

C Correlations

		Hus1 Female	*Hus1* Male
Hus1 Female	Pearson correlation	1	0.985**
	Sig. (2-tailed)		0.000
	N	9	9
Hus1 Male	Pearson correlation	0.985**	1
	Sig. (2-tailed)	0.000	
	N	9	9

FIG. 2. The mRNA levels of *Rad9, Rad1* and *Hus1* genes were strongly correlated in various normal mouse tissues between male and female mice. Panel A: Correlation coefficient of expression levels of *Rad9* gene between the male and female mice. Panel B: Correlation coefficient of expression levels of *Rad1* gene between the male and female mice. Panel C: Correlation coefficient of expression levels of *Hus1* gene between the male and female mice.

not in all tissues. We also performed experiments on 6 tissues from male mice (liver, lung, small intestine, skeletal muscle, testis and skin). The expressions of 5 tissues (excluding the testis) after irradiation were similar to those in the corresponding tissues from female mice (Supplementary Fig. S2; http://dx.doi.org/10.1667/RR13781.1.S1). The expression pattern of testis was also similar to that of ovary although the induction scales of the expressions of the 3 genes in the testis were larger than those in the ovary.

Induced expression of *Rad9* characteristically peaked at 2 and 12 h after irradiation in all the tissues except the spleen and skeletal muscle (Fig. 3A). This pattern also occurred for *Hus1* in skin tissue and less typically in heart tissue, but not in the other tissues (Fig. 3C). *Hus1* expression peaked at 2 h after irradiation in all tissues except the small intestine and skeletal muscle, however *Hus1* expression also reached very high levels at 48 h postirradiation in many tissues. *Rad1* expressions were not significantly altered, but there was a slight enhancement in lung, heart and liver tissues at 48 h after irradiation (Fig. 3B). Although both *Rad9* and *Hus1* had significantly altered expression levels after irradiation in some tissues at several overlapping time points, there was no correlation between their changes (data not shown here). These results suggest that the mechanisms of radiation-induced gene regulation are different for the three genes.

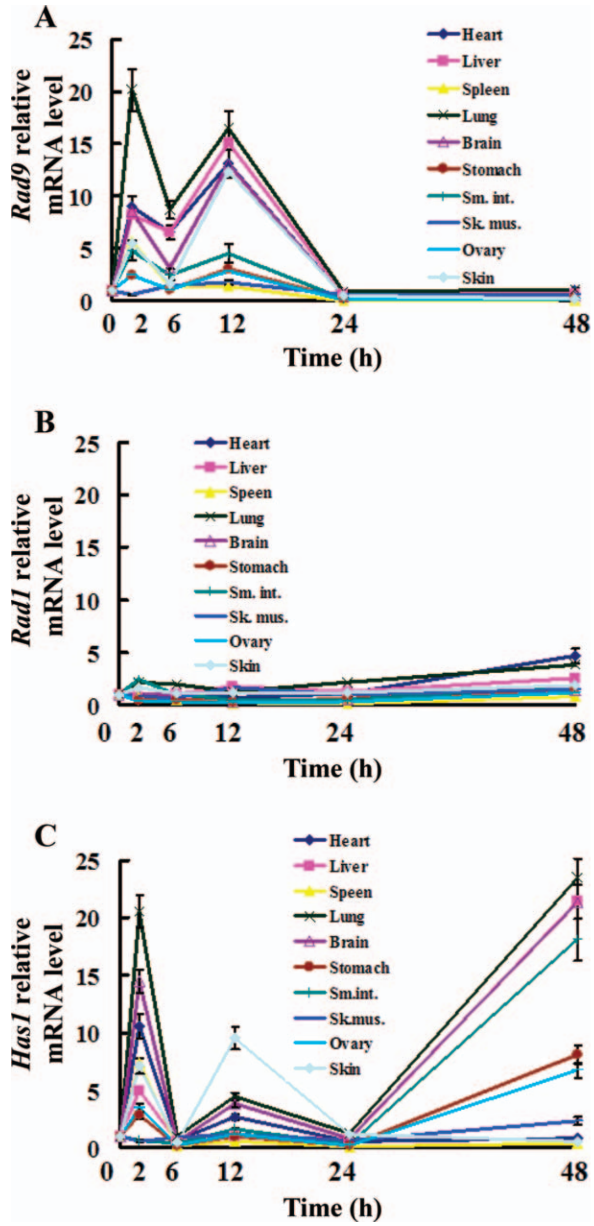


FIG. 3. The alterations of the mRNA of *Rad9, Hus1* and *Rad1* genes in female 129 mouse tissues (heart, liver, spleen, lung, brain, stomach, small intestine, skeletal muscle, ovary and skin) in response to gamma irradiation. The mRNA levels were measured by quantitative real-time PCR. Panel A: The mRNA levels of *Rad9*. Panel B: The mRNA levels of *Rad1*. Panel C: The mRNA levels of *Hus1*. Sm. int. = small intestine; Sk. mus. = skeletal muscle.

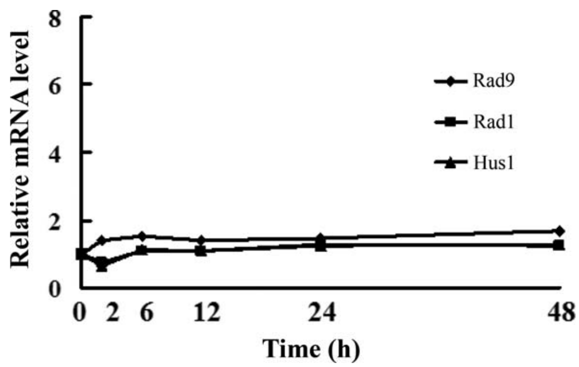


FIG. 4. The alterations of the mRNA of *Rad9, Hus1* and *Rad1* genes in mouse liver cells (NCTC 1469).

Furthermore, our data indicates that for each of the three genes, there was not a single consistent trend in their mRNA induction response to gamma irradiation across all the tissues. In addition, even though there were distinct dominant features in each of the three genes such as the peaked expressions of *Rad9* at 2 and 12 h, and of *Hus1* at 48 h, as well as the minimally induced alterations of *Rad1* expression, the extent of these changes varied significantly among these tissues. These data suggest that there is not a single mechanism of the radiation-induced regulation of gene expression for any one of the three genes that fit with all the tissues examined.

Responses of Rad9, Hus1 and Rad1 Genes at mRNA Levels of In Vitro Cultured Mouse Liver Cells to Ionizing Radiation

The two peaks of *Rad9* mRNA in 8 of the 10 tissues, and of *Hus1* mRNA in 3 of the 10 tissues, which were induced by 10 Gy gamma irradiation are particularly unusual, because radiation-induced gene upregulation of cells is more often single-peaked (*43–45*). To determine if these peculiar responses of the *Rad9* and *Hus1* genes to irradiation were tissue environment-dependent, mouse liver cells (NCTC 1469) cultured in dishes were gamma irradiated with 10 Gy and the mRNA levels of the three genes were then examined using qPCR. In contrast to the dramatic changes of *Rad9* and *Hus1* mRNA levels in the mouse liver tissue, the mRNA levels of these two genes were altered less than onefold. And although the *Rad1* mRNA level in the mouse liver tissue was not very responsive to radiation, it did change a bit more than the cultured mouse liver cells [Fig. 4 and Supplementary Fig. S3 (http://dx.doi.org/10.1667/RR13781.1.S1)]. These data suggest that the organization of tissues significantly modifies cellular regulation of the mRNA levels in response to radiation.

Changes of Rad9 and Hus1 Protein Levels in Mouse Liver and Heart Tissues and Cultured Mouse Liver Cells in Response to Ionizing Radiation

Biological functions of coding genes are carried out by their proteins. Although the mRNA levels of the three genes did not change in a coordinated manner in response to exposure to ionizing radiation, as shown above, their protein levels might still be altered in a coordinated manner so the 9-1-1 complex could be formed and carry out its functions efficiently. Using Western blotting, we measured protein levels of Rad9 and Hus1 in liver and heart tissues, and in cultured liver cells before and after 10 Gy gamma irradiation. Interestingly, Rad9 protein levels were sequentially peaked, dipped and enhanced/peaked again in the two tissues and the cultured cells, although the timing of when the protein levels peaked and dipped were different from their mRNA levels [Figs. 5 and 6; Supplementary Figs. S4 and S5 (http://dx.doi.org/10.1667/RR13781.1.S1)]. In contrast to Rad9, the protein level alterations of Hus1 after irradiation followed three different patterns (Figs. 5 and 6; Supplementary Figs. S4 and S5). The Hus1 protein level in mouse liver tissue peaked at 12 h, dipped slightly at 24 h and then rose again. In the heart tissue, the Hus1 protein level changed only minimally during the 48 h period after irradiation. In the cultured liver cells the Hus1 protein level quickly increased within the first 12 h, then slowly continued to enhance until the end of the experiment at 48 h. These data suggest that the Rad9 and Hus1 protein levels do not change in a coordinated manner in mouse liver and heart tissues, as well as in cultured mouse liver cells. These data also indicate that Hus1 protein levels change differently in liver and heart tissues in response to exposure to ionizing radiation.

We also assayed the Rad9 and Hus1 proteins in the other tissues, but the specificity was not sufficient enough to provide clear results. Other commercial antibodies were tested, but none of them worked for this study. In addition, we tested many commercial Rad1 antibodies and several batches of self-made Rad1 antibodies, and none of these provided sufficiently clear results.

DISCUSSION

Rad9, Hus1 and Rad1 form a ring-like trimer complex that has been suggested to play a role in cell cycle checkpoint control (*14, 15, 46*) and various forms of DNA repair (*47*). In this study, the mRNA levels of *Rad9, Hus1* and *Rad1* were found to be well correlated across 10 tissues of 129 mice under sham-irradiated conditions [Fig. 1 and Supplementary Fig. S1 (http://dx.doi.org/10.1667/RR13781.1.S1)], suggesting that the mRNA levels of the three genes are maintained in an orchestrated manner perhaps so the 9-1-1 complex can be formed efficiently and maintain genome integrity in normal physiological conditions. Interestingly, the mRNA levels in spleen, ovary/testis and lung were significantly higher than in the other tissues. The high mRNA levels of the three genes in spleen and ovary/testis are consistent with their roles in cell cycle checkpoint and DNA repair of genes needed for formation of antibodies in spleen and meiosis in ovary/testis that utilize DNA breakage/repair processes (*48–51*). The high

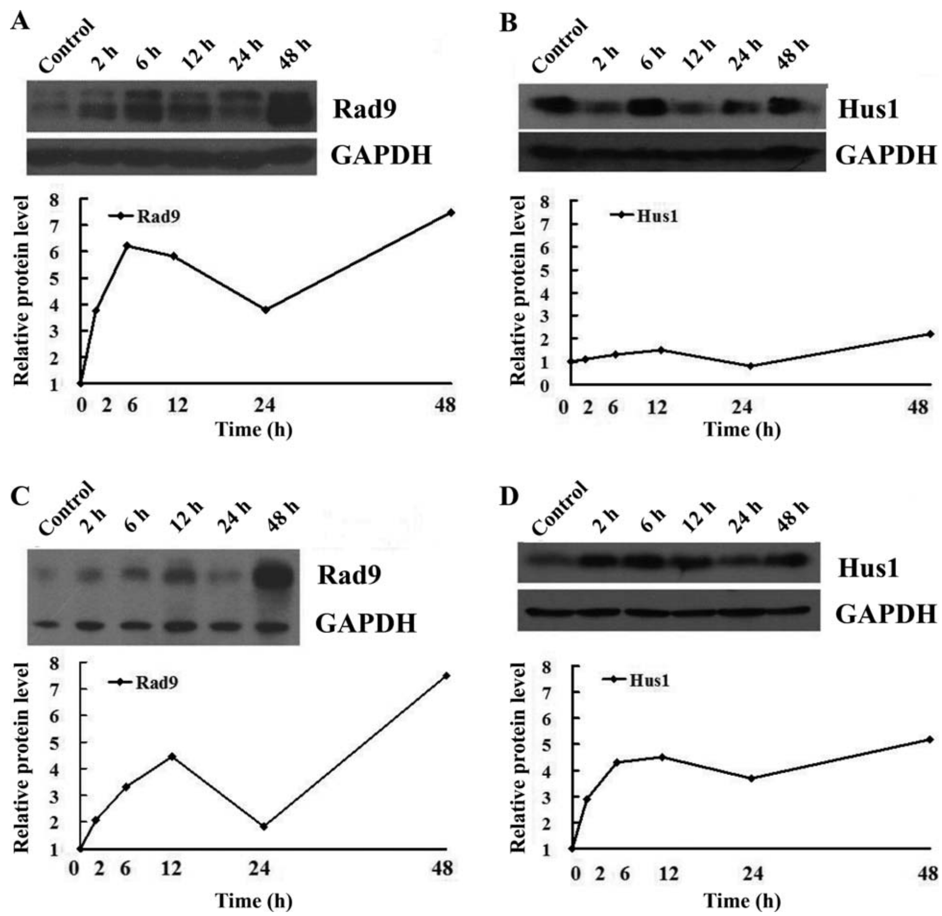


FIG. 5. Western blotting analysis of Rad9 and Hus1 protein levels in irradiated mouse heart and liver tissue: Rad9 in heart (panel A); Hus1 in heart (panel B); Rad9 in liver (panel C); and Hus1 in liver (panel D). In each set of the above experiments, panels A–D, the first lane is the protein level in the sham-irradiated control and the remaining five lanes are the protein levels in the liver tissue harvested at 2, 6, 12, 24 and 48 h after irradiation. The top portion of the panels are scanned Western blots and the line graphs are the corresponding density quantification result (presented as protein level versus time).

mRNA levels of the three genes in lung were surprising but perhaps understandable, since oxygen causes DNA lesions (*52, 53*). Lung alveolar cells are exposed to a much higher concentration of oxygen than the other normal tissues, i.e., partial pressures of alveolar air and normal tissues are 104 mmHg and 30 mmHg, respectively (*54–57*). *Rad9* has been shown to play an important role in antioxidation in mouse skin keratinocytes (*20*). The high mRNA levels of the three genes in the lung tissue suggest that lung cells, particularly the alveolar cells, are exposed to elevated oxidative stress and need a mechanism to maintain cellular genome integrity with perphaps *Rad9, Hus1* and *Rad1* playing an important role. The differential levels of mRNA measured by qPCR generally agree with the Northern blot results previously reported, although the mouse Hus1 and Rad1 Northern blot results were not quantitatively analyzed in the original reports, and all the RNA species (with various sizes on the blots) of the 9-1-1 complex component genes and the control genes β-*actin* or *Gapdh* would need to be added and the data reanalyzed to reach a conclusion (*58–60*).

Notably, *Rad9* mRNA levels were significantly higher than *Rad1* and *Hus1* mRNA levels in the lung and spleen tissues, and this contributes to the lower correlations between *Rad9* and *Rad1* or *Hus1* mRNA levels than the correlations between *Rad1* and *Hus1* mRNA levels [Fig. 1 and Supplementary Fig. S1 (http://dx.doi.org/10.1667/RR13781.1.S1)]. The current general view on the working mechanism of Rad9, Rad1 and Hus1 proteins is that they form a heterotrimer ring and function in DNA repair and cell cycle checkpoint by this ring structure form. The question is whether the three components also play roles in a ring-independent individual form. The three genes manifest some distinct features. $Rad9^{+/-}$ mouse keratinocytes grow significantly slower than $Rad1^{+/-}$ mouse keratinocytes (*22, 41*). Rad1 protein exists in a free single-molecular form besides the 9-1-1 complex form in human leukemia K562 (*37*) and human embryonic kidney 293 cells (our unpublished data). More studies are needed to specify the exact molecular role(s) of individual molecules of Rad9, Rad1 and/or Hus1 proteins in cells.

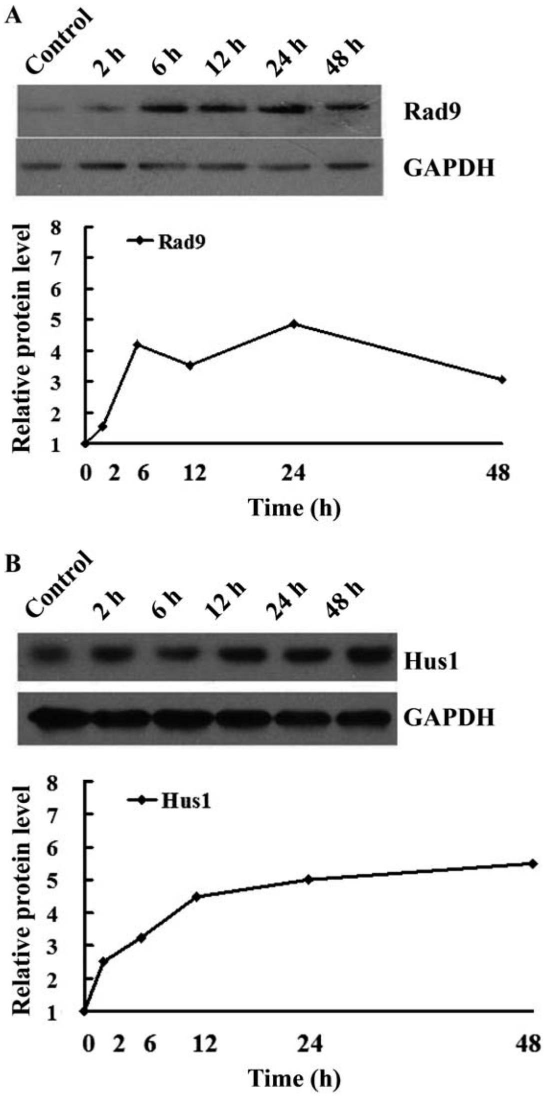


FIG. 6. Western Blotting analysis of Rad9 and Hus1 protein levels in irradiated mouse liver cells (NCTC 1469): Rad9 (panel A); Hus1 (panel B).

The mRNA levels of the three genes in response to 10 Gy gamma irradiation were altered very differently in timing and extent (Fig. 3). *Rad9* mRNA levels in many tissues peaked at 2 and/or 12 h after irradiation, *Hus1* mRNA levels in many tissues peaked at 2, 12 and/or 24 h after irradiation, while *Rad1* mRNA levels were minimally changed except for a very slight enhancement in lung, heart and liver tissues 48 h after irradiation (Fig. 3B). The distinct patterns of the mRNA level changes for the three genes in response to exposure to gamma radiation suggest that the mRNA levels are regulated by different mechanisms. mRNA levels can be influenced by transcription and mRNA stabilization. The good correlation among the mRNA levels across these tissues under normal conditions [Fig. 1A and Supplementary Fig. S1 (http://dx.doi.org/10.1667/RR13781.1.S1)], suggests that DNA lesion levels are monitored by cells and the signals are transmitted to the transcription machinery and/or mRNA stabilizing factors. For example, the physiological programmed DNA lesions and repair in spleen, testis and ovary are sensed, and high levels of mRNA of the three genes are maintained for repair in the cells in these tissues. The fact that the mRNA level changes of the three genes at various time points after irradiation are not correlated [Fig. 3 and Supplementary Fig. S2 (http://dx.doi.org/10.1667/RR13781.1.S1)] suggests that the DNA lesion level is not the only element sensed by transcription machinery and/or mRNA stabilizing factors, and the other element(s) that influence the mRNA level play different roles in controlling the mRNA levels of the three different genes. Since the patterns of mRNA changes for the three genes were different from each other, at least some other of the mRNA controlling elements should be on these genes. Therefore, there should be at least two mechanisms controlling the mRNA levels; one is dominant under physiological conditions, while the other exerts its influence mainly when the cells undergo stresses such as irradiation. Although the radiation-induced mRNA levels of Rad9 and Hus1were highly dynamic in the liver, they barely changed in the *in vitro* cultured liver cells after irradiation [Fig. 4 and Supplementary Fig. S3 (http://dx.doi.org/10.1667/RR13781.1.S1)], suggesting that signals outside of cells greatly influence transcription machinery and/or mRNA stabilizing factors through the aforementioned second mechanism.

The differences of mRNA induction of the three genes did not necessarily transfer to the unbalanced protein levels and inefficiency of the formation of the 9-1-1 complex. Notably, there exists free Rad1 protein in untreated K526 (*37*) and 293 cells (our unpublished data), and the extra free Rad1 might form the 9-1-1 complex with newly synthesized Rad9 and Hus1. To clarify the above question on the balanced protein concentrations of the three genes, we attempted to detect the three proteins in mouse tissues before and after irradiation. However, all Western blotting assays using commercial and self-made mouse Rad1 antibodies failed to generate useful data. We were also unable to obtain sufficient Western blotting data on Rad9 and Hus1 of all the mouse tissues except for the liver and heart [Figs. 5 and 6; Supplementary Figs. S4 and S5 (http://dx.doi.org/10.1667/RR13781.1.S1); unpublished data]. However, we are still able to make three comparisons using the protein data and the above mRNA data: 1. protein level changes between Rad9 and Hus1; 2. protein level changes between heart and liver; 3. protein level changes and mRNA level changes. These are further discussed below.

In the liver, both Rad9 and Hus1 protein levels peaked at 12 h and reached even higher values at 48 h; Rad9 protein level reached a concave low point at 24 h while Hus1 protein level was only slightly lower before rising again [Fig. 5C and D; Supplementary Fig. S4C and D (http://dx.doi.org/10.1667/RR13781.1.S1)]. In the heart tissue, Rad9 protein level peaked at 6 h and reached a concave point at

24 h before rising again to the highest value in the experiment time range. Meanwhile, the change of Hus1 protein level was much less dynamic, and its highest value only doubled that of the nonirradiated control [Fig. 5A and B; Supplementary Fig. S4A and B (http://dx.doi.org/10.1667/RR13781.1.S1)]. These results suggest that the Rad9 and Hus1 protein levels were not altered in an orchestrated manner in the heart, and changed at most in a loosely coordinated manner in the liver.

As for comparisons of the radiation-induced changes of the Rad9 and Hus1 protein levels between liver and heart, there are two interesting points. First, the Rad9 protein level reached the first peak in the heart and liver at 6 and 12 h, respectively. Second, the Hus1 protein level changed in a highly dynamic manner in the liver, but only slightly altered in the heart during the 48 h after irradiation [Fig. 5 and Supplementary Fig. S4 (http://dx.doi.org/10.1667/RR13781.1.S1)]. In short, either the timing (Rad9) or extent (Hus1) of the protein level alteration was different in liver and heart tissues, indicating that the two proteins are regulated differently in the two tissues, or simply that this reflects distinct intracellular environments in the two tissues that influence the protein levels in response to the same dose of ionizing radiation.

The dynamic changes of Rad9 and Hus1 protein levels in liver and heart tissues were obviously different from those of the mRNA levels after irradiation (Figs. 3 and 5). *Rad9* mRNA levels peaked in both the heart and liver at 2 and 12 h while the protein levels in the heart peaked at 6 and 48 h, and in the liver at 12 and 48 h, thus the rise of the Rad9 protein level followed the trend of the induced mRNA level. The *Hus1* mRNA level peaked both at 2 and 12 h the in heart, and only peaked at 2 h to a level less than that in the liver during the 24 h period after irradiation. However, the Hus1 protein levels changed much more dramatically in the liver than in the heart during the 24 h period. The above data indicates that the Hus1 protein induction in the heart does not follow the trend of its mRNA level, suggesting that Hus1 protein level in the heart maybe mainly regulated at translation and/or protein degradation, and that the Hus1 protein levels are regulated differently in the liver and heart.

The two or three peaks of the *Rad9* and *Hus1* mRNA levels in some mouse tissues after irradiation were quite unique (Fig. 3), and this phenomenon prompted us to investigate if there was a similar trend for *in vitro* cultured mouse liver hepatocytes (NCTC 1469). In contrast to the highly dynamic alterations in liver tissues, the *Rad9, Rad1* and *Hus1* mRNA levels barely changed after irradiation [Fig. 4 and Supplementary Fig. S3 (http://dx.doi.org/10.1667/RR13781.1.S1)], suggesting that the liver cells in tissue respond to radiation very differently from those in culture. Despite this difference in radiation-induced mRNA levels, the changes in the *Rad9* and *Hus1* protein levels between cultured liver cells and cells in the liver were somewhat close to each other even though the timings and extents were not the same. These data indicate that regulation of translation or protein degradation may play an important role in determining Rad9 and Hus1 protein levels.

The differential radiation-induced mRNA or protein levels of the three genes do not challenge the 9-1-1 complex structure and its potential physiological roles. However, this does not appear to reconcile with the current proposal that the complex plays an important role in dealing with severe DNA damage caused by high-dose ionizing irradiation. Thus far there has been no direct evidence that supports this proposal. An experiment, where one mutant component protein (such as mutant Rad1) unable to physically interact with the other two components (such as Rad9 and Hus1) still confers resistance to a high dose of radiation, would help clarify if the complex plays a role in resistance to a high dose of radiation or not.

The 9-1-1 complex is not the only complex in which component genes are expressed in a correlated manner among different tissues under normal conditions and expressed in an uncorrelated manner under genotoxic stresses. The Ku70/Ku80 complex plays a critical role in DNA double-strand nonhomologous end joining (NHEJ). The mRNA levels of Ku70 and Ku80 undergo a dynamic and correlated change during zebrafish embryogenesis (*61, 62*). The expression levels of Ku70 and Ku80 in human rectal intestine (*63*), breast, cervix (*64*) epithelium cells (*65*) (immunostaining) and fibroblasts (*66*) (Northern blotting) were also reported to be correlated. However, their expressions are not always correlated under genotoxic stress conditions, such as the Ku70 and Ku80 protein levels in livers of mice injected with dimethylnitrosamine (*67*) and in mouse B cells during B cell differentiation (*68*). Consistently, Ku70 and Ku80 manifest different functions in T cell differentiation (*69–71*) and apoptosis (*72–74*), although both are required for NHEJ. It is likely that the correlated and uncorrelated expressions correspond to functions requiring both components and only one component, respectively.

In summary we found: 1. A good correlation of mRNA levels among the three genes *Rad9*, *Hus2* and *Rad1*, which indicates that they function coordinately under normal physical conditions *in vivo*, probably through the formation of the 9-1-1 complex; 2. Tissue environment plays a profound role in the regulation of the mRNA and protein levels of the three genes in response to exposure to ionizing radiation; and 3. There is no orchestration in either mRNA or protein level change in response to 10 Gy gamma irradiation, suggesting that either the genes do not respond in a coordinated manner to the high dose of radiation not in mechanisms evolved through adapting environment, or the individual components play independent and diverse roles in high-dose radiation response. It is evident that further experimental studies are needed to investigate these possibilities.

SUPPLEMENTARY INFORMATION

Fig. S1. The expressions of *Rad9, Rad1* and *Hus1* genes in male 129 mouse tissues (heart, liver, spleen, lung, brain, stomach, small intestine, skeletal muscle, testis and skin). The expressions were measured by quantitative real-time PCR. Panel A: The mRNA levels of *Rad9, Rad1* and *Hus1* genes in various normal mouse tissues. The expression levels in spleen, lung and testis were significantly higher than in the other seven tissues. Data are presented as ratios relative to the expression of GAPDH and are normalized by average levels of the 10 tissues. Error bars represent standard deviations of three independent experiments. Sm. Int. = small intestine; Sk. Mus. = skeletal muscle. Panel B: Correlation coefficient of expression levels of *Rad9, Rad1* and *Hus1* genes. The mRNA levels of *Rad9, Rad1* and *Hus1* genes showed statistically significant correlations in various normal mouse tissues, especially between *Rad1* and *Hus1*.

Fig. S2. The alterations of the mRNA of *Rad9*, *Hus1* and *Rad1* genes in male 129 mouse tissues (liver, lung, small intestine, skeletal muscle, testis and skin) in response to gamma irradiation. This data, from a partially duplicated experiment shown in Fig. 3, here uses male mice instead of the female mice shown in Fig. 3.

Fig. S3. The alterations of the mRNA of *Rad9, Hus1* and *Rad1* genes in mouse liver cells (NCTC 1469). This is the data of a duplicated experiment of that shown in Fig. 4.

Fig. S4. Western blotting analysis of Rad9 and Hus1 protein levels in irradiated mouse heart and liver tissue. This is the data of a duplicated experiment of that shown in Fig. 5.

Fig. S5. Western Blotting analysis of Rad9 and Hus1 protein levels in irradiated mouse liver cells (NCTC 1469): Rad9 (panel A); Hus1 (panel B). This is the data of a duplicated experiment of that shown in Fig. 6.

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